

## TRANSMITTAL LETTER TO THE UNITED STATES

DESIGNATED/ELECTED OFFICE (DO/EO/US)

CONCERNING A FILING UNDER 35 U.S.C. 371

PA-9848

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

To be assigned 09/869629

INTERNATIONAL APPLICATION NO.

PCT/GB99/04410

INTERNATIONAL FILING DATE

December 23, 1999

PRIORITY DATE CLAIMED

December 30, 1998

TITLE OF INVENTION

NMR Spectroscopic In Vitro Assay Using Hyperpolarization

APPLICANT(S) FOR DO/EO/US

P. Knox, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☒ is attached hereto (required only if not communicated by the International-Bureau).
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - a. ☐ is attached hereto.
  - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

## Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

copy of this transmittal letter for charging purposes

copy of the International Application as published by the International Bureau

return postcard

APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.53(b))

09/069629

NATIONAL APPLICATION NO.

PCT/GB99/04410

ATTORNEY'S DOCKET NUMBER

PA-9848

The following fees are submitted:

CALCULATIONS PTO USE ONLY

**BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5) ) :**

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1000.00
- ☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

\$860.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

\$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	29 - 20 =	9	x \$18.00
Independent claims	1 - 3 =	0	x \$80.00

\$162.00

\$0.00

Multiple Dependent Claims (check if applicable). ☐

\$0.00

**TOTAL OF ABOVE CALCULATIONS =**

\$1,022.00

☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.

\$0.00

**SUBTOTAL =**

\$1,022.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).

\$0.00

**TOTAL NATIONAL FEE =**

\$1,022.00

Fees for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). ☐

\$0.00

**TOTAL FEES ENCLOSED =**

\$1,022.00

Amount to be:  
refunded \$  
charged \$

- ☐ A check in the amount of \_\_\_\_\_ to cover the above fees is enclosed.
- ☒ Please charge my Deposit Account No. 500-588 in the amount of \$1,022.00 to cover the above fees. A duplicate copy of this sheet is enclosed.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 500-588. A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Royal N. Ronning, Jr.  
Amersham Pharmacia Biotech, Inc.  
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(732) 457-8423

SIGNATURE

Royal N. Ronning, Jr.

NAME

32,529

REGISTRATION NUMBER

June 28, 2001

DATE

PA-9848

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Application of: P. Knox, et al.      Group Art Unit: To be assigned  
Serial Number: To be assigned      Examiner: To be assigned  
Filing Date: June 28, 2001  
Title: NMR Spectroscopic In Vitro Assay Using Hyperpolarization

**FIRST PRELIMINARY AMENDMENT**

Honorable Assistant Commissioner of Patents  
Box New Patent Application  
Washington, D.C. 20231

Sir:

Please consider the following amendments and remarks in connection with the prosecution of the captioned application, which is a filing under 35 U.S.C. § 371 and claims priority to international application number PCT/GB99/04410 filed December 23, 1999, application number 9828852.5 filed in Great Britain on December 30, 1998, and application number 9918096.0 filed in Great Britain on August 2, 1999.

## **In the Claims**

Please amend page 21, line 1 as follows:

### **[C L A I M S]**

#### **What is claimed is:**

Please amend claim 4 as follows:

4. (once amended) The method of [any of claims 1 to 3]claim 1, wherein the assay reagent is a compound which contains an artificially high concentration of an NMR active nucleus.

Please amend claim 6 as follows:

6. (once amended) The method of [any of claims 1 to 5]claim 1, wherein the assay reagent is an organic compound comprising one or more NMR active nuclei associated with a bond which is broken during the course of the assay.

Please amend claim 8 as follows:

8. (once amended) The method of [any claim 1-7]claim 1, wherein the assay reagent is analysed repeatedly in step c) at known time intervals so as to generate information about a change with time of the assay reagent.

Please amend claim 9 as follows:

9. (once amended) The method of [any one claim 1 to 8]claim 1, wherein the [asseay]assay reagent is a Nucleotide, or nucleotide analogue, polynucleotide, amino acid analogue, polypeptide or protein.

Please amend claim 10 as follows:

10. (once amended) The method of [any one of claims 1 to 9]claim 1, wherein the assay is a nucleic acid hybridisation assay.

Please amend claim 11 as follows:

11. (once amended) The method of [any one of claims 1 to 10]claim 1, wherein the assay is a binding assay.

Please amend claim 12 as follows:

12. (once amended) The method of [claims 1 to 11]claim 1, wherein the assay reagent is a compound specifically labelled with at least one NMR active nucleus and the assay reagent is administered to a micro-organism, macro-organism or cultured cells, cellular metabolites or an excretion product of the assay reagent are hyperpolarised and analysed by nuclear magnetic resonance spectroscopy, nuclear magnetic resonance imaging or both.

Please amend claim 13 as follows:

13. (once amended) The method of [claims 1 to 12]claim 1, wherein the assay is a binding study performed using micro-organisms or cultured cells

Please amend claim 14 as follows:

14. (once amended) The method of [claims 1 to 13]claim 1 wherein the hyperpolarisation transfer is repeated to enhance the signal-to-noise ratio.

Please amend claim 15 as follows:

15. (once amended) The method of [claim 1 to 14]claim 1 wherein the shortening

effect as expressed by the improvement of signal-to-noise per unit time is a factor of 10 or more compared to known assay techniques without hyperpolarisation.

Please amend claim 16 as follows:

16. (once amended) The method of [claims 1 to 15]claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer from a hyperpolarised noble gas, or a mixture of hyperpolarised noble gases.

Please amend claim 19 as follows:

19. (once amended) The method of [claims 16 to 18]claim 16 wherein the hyperpolarisation is transferred by a hyperpolarised noble gas in solution and wherein the viscosity of the solution is at least 1000 mPs.

Please amend claim 20 as follows:

20. (once amended) The method of [claims 1 to 15]claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer at a temperature of 4.2 K or less in the presence of a magnetic field of at least 1 T.

Please amend claim 21 as follows:

21. (once amended) The method of [claims 1 to 15]claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer using dynamic nuclear polarisation.

Please amend claim 22 as follows:

22. (once amended) The method of [claims 1 to 15]claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by para hydrogen induced polarisation.

Please amend claim 23 as follows:

23. (once amended) The method of [claims 1 to 15]claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out with the spin refrigeration technique.

Please amend claim 24 as follows:

24. (once amended) The method of [claims 1 to 23]claim 1, wherein more than one



assay is multiplexed and monitored by NMR spectroscopy and/or NMR imaging.

Please amend claim 25 as follows:

25. (once amended) The method of [claims 1 to 24]claim 1 wherein the assay is performed in a multiwell or multispot assay array.

Please amend claim 26 as follows:

26. (once amended) The method of [claims 1 to 25]claim 1 wherein step c) is performed by examining the assay reagent using both NMR spectroscopy to obtain more than one spectrum, and magnetic resonance imaging to obtain one or more discrete spectral location, and repeating the examination at least once so as to obtain quantitative information about kinetic or time-dependant alteration in chemistry, environment or structure of the assay reagent.

Please amend claim 27 as follows:

27. (once amended) The method of [claim 1 to 26]claim 1, wherein step c) is performed in an aerosol or flow-through device applied to aerosol droplets where the well, surface or container is used to contain the assay reagent.

Please amend claim 28 as follows:

28. (once amended) An *in vitro* assay kit for carrying out the assay method as defined in claim 1 [to 27 ]which comprises: one or more assay reagents each containing at least one NMR active nucleus contained in a well or vial or other suitable container for carrying out the hyperpolarisation of step (b) of claim 1.

Please amend claim 29 as follows:

29. (once amended) The *in vitro* kit of claim 28 where the NMR analysis of step (c) [of claim 1] is carried out in the same well, vial or container as the hyperpolarisation transfer is carried out.

### **Remarks**

Claims 1-29 are pending in the instant application. Applicants have amended claims 4, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, and 29 to more fully conform with U.S. practice and to delete multiple dependencies. A version of the claims marked up to show the amendments, as well as a clean version of the claims encompassing the amendments, is attached hereto.

Applicants respectfully assert that all amendments are fairly based on the specification, and respectfully request their entry.

Applicants believe that the claims, as amended, are in allowable form, and earnestly solicit the allowance of claims 1-29.

Respectfully submitted,

A handwritten signature in black ink, appearing to read "Royal N. Ronning, Jr.", written in a cursive style.

Royal N. Ronning, Jr. 32,529  
Attorney for Applicants

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## Claims (marked-up version showing amendment(s))

### [C L A I M S]

#### What is claimed is:

4. (once amended) The method of [any of claims 1 to 3]claim 1, wherein the assay reagent is a compound which contains an artificially high concentration of an NMR active nucleus.
6. (once amended) The method of [any of claims 1 to 5]claim 1, wherein the assay reagent is an organic compound comprising one or more NMR active nuclei associated with a bond which is broken during the course of the assay.
8. (once amended) The method of [any claim 1-7]claim 1, wherein the assay reagent is analysed repeatedly in step c) at known time intervals so as to generate information about a change with time of the assay reagent.
9. (once amended) The method of [any one claim 1 to 8]claim 1, wherein the [asseay]assay reagent is a Nucleotide, or nucleotide analogue, polynucleotide, amino acid analogue, polypeptide or protein.
10. (once amended) The method of [any one of claims 1 to 9]claim 1, wherein the

assay is a nucleic acid hybridisation assay.

11. (once amended) The method of [any one of claims 1 to 10]claim 1, wherein the assay is a binding assay.
12. (once amended) The method of [claims 1 to 11]claim 1, wherein the assay reagent is a compound specifically labelled with at least one NMR active nucleus and the assay reagent is administered to a micro-organism, macro-organism or cultured cells, cellular metabolites or an excretion product of the assay reagent are hyperpolarised and analysed by nuclear magnetic resonance spectroscopy, nuclear magnetic resonance imaging or both.
13. (once amended) The method of [claims 1 to 12]claim 1, wherein the assay is a binding study performed using micro-organisms or cultured cells
14. (once amended) The method of [claims 1 to 13]claim 1 wherein the hyperpolarisation transfer is repeated to enhance the signal-to-noise ratio.
15. (once amended) The method of [claim 1 to 14]claim 1 wherein the shortening effect as expressed by the improvement of signal-to-noise per unit time is a factor of 10 or more compared to known assay techniques without hyperpolarisation.

16. (once amended) The method of [claims 1 to 15]claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer from a hyperpolarised noble gas, or a mixture of hyperpolarised noble gases.
19. (once amended) The method of [claims 16 to 18]claim 16 wherein the hyperpolarisation is transferred by a hyperpolarised noble gas in solution and wherein the viscosity of the solution is at least 1000 mPs.
20. (once amended) The method of [claims 1 to 15]claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer at a temperature of 4.2 K or less in the presence of a magnetic field of at least 1 T.
21. (once amended) The method of [claims 1 to 15]claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer using dynamic nuclear polarisation.
22. (once amended) The method of [claims 1 to 15]claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by para hydrogen induced polarisation.

23. (once amended) The method of [claims 1 to 15]claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out with the spin refrigeration technique.
24. (once amended) The method of [claims 1 to 23]claim 1, wherein more than one assay is multiplexed and monitored by NMR spectroscopy and/or NMR imaging.
25. (once amended) The method of [claims 1 to 24]claim 1 wherein the assay is performed in a multiwell or multispot assay array.
26. (once amended) The method of [claims 1 to 25]claim 1 wherein step c) is performed by examining the assay reagent using both NMR spectroscopy to obtain more than one spectrum, and magnetic resonance imaging to obtain one or more discrete spectral location, and repeating the examination at least once so as to obtain quantitative information about kinetic or time-dependant alteration in chemistry, environment or structure of the assay reagent.
27. (once amended) The method of [claim 1 to 26]claim 1, wherein step c) is performed in an aerosol or flow-through device applied to aerosol droplets where the well, surface or container is used to contain the assay reagent.
28. (once amended) An *in vitro* assay kit for carrying out the assay method as defined

in claim 1 [to 27 ]which comprises: one or more assay reagents each containing at least one NMR active nucleus contained in a well or vial or other suitable container for carrying out the hyperpolarisation of step (b) of claim 1.

29. (once amended) The *in vitro* kit of claim 28 where the NMR analysis of step (c) [of claim 1] is carried out in the same well, vial or container as the hyperpolarisation transfer is carried out.

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## Claims (clean version encompassing amendments)

### What is claimed is:

1. An *in vitro* assay method which comprises:
  - a) using an assay reagent containing at least one NMR active nucleus to perform an assay, and
  - b) hyperpolarising at least one NMR active nucleus of the assay reagent;  
wherein steps (a) and (b) are performed simultaneously or sequentially in either order, and
  - c) analysing the assay reagent and/or the assay by NMR and
  - d) optionally using the NMR data obtained in step c) to generate further assay result(s).
2. The method of claim 1 wherein the NMR active nucleus  $^{15}\text{N}$ ,  $^{19}\text{F}$ ,  $^{31}\text{P}$ ,  $^1\text{H}$ ,  $^{29}\text{Si}$  and/or  $^{13}\text{C}$ .
3. The method of claim 2, wherein the NMR active nucleus is  $^{15}\text{N}$  or  $^{13}\text{C}$ .
4. (once amended) The method of claim 1, wherein the assay reagent is a compound which contains an artificially high concentration of an NMR active nucleus.

5. The method of claim 4, wherein the assay reagent contains an artificially high concentration in 1-10 defined positions.
6. (once amended) The method of claim 1, wherein the assay reagent is an organic compound comprising one or more NMR active nuclei associated with a bond which is broken during the course of the assay.
7. The method of claim 6, wherein the assay reagent contains two or more NMR active nuclei and each NMR active nucleus produces a distinct NMR spectrum and when the assay method is performed, it results in changes to the chemical and/or physical environment of the nucleus and this is mirrored by spectral changes which can be monitored.
8. (once amended) The method of claim 1, wherein the assay reagent is analysed repeatedly in step c) at known time intervals so as to generate information about a change with time of the assay reagent.
9. (once amended) The method of claim 1, wherein the assay reagent is a Nucleotide, or nucleotide analogue, polynucleotide, amino acid analogue, polypeptide or protein.
10. (once amended) The method of claim 1, wherein the assay is a nucleic acid

hybridisation assay.

11. (once amended) The method of claim 1, wherein the assay is a binding assay.
12. (once amended) The method of claim 1, wherein the assay reagent is a compound specifically labelled with at least one NMR active nucleus and the assay reagent is administered to a micro-organism, macro-organism or cultured cells, cellular metabolites or an excretion product of the assay reagent are hyperpolarised and analysed by nuclear magnetic resonance spectroscopy, nuclear magnetic resonance imaging or both.
13. (once amended) The method of claim 1, wherein the assay is a binding study performed using micro-organisms or cultured cells
14. (once amended) The method of claim 1 wherein the hyperpolarisation transfer is repeated to enhance the signal-to-noise ratio.
15. (once amended) The method of claim 1 wherein the shortening effect as expressed by the improvement of signal-to-noise per unit time is a factor of 10 or more compared to known assay techniques without hyperpolarisation.
16. (once amended) The method of claim 1 where the hyperpolarisation of the NMR

active nucleus of the assay reagent is carried out by polarisation transfer from a hyperpolarised noble gas, or a mixture of hyperpolarised noble gases.

17. The method of claim 16 wherein the noble gas is  $^{129}\text{Xe}$ .
18. The method of claim 16 wherein the noble gas is  $^3\text{He}$ .
19. (once amended) The method of claim 16 wherein the hyperpolarisation is transferred by a hyperpolarised noble gas in solution and wherein the viscosity of the solution is at least 1000 mPs.
20. (once amended) The method of claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer at a temperature of 4.2 K or less in the presence of a magnetic field of at least 1 T.
21. (once amended) The method of claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer using dynamic nuclear polarisation.
22. (once amended) The method of claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by para hydrogen induced polarisation.

23. (once amended) The method of claim 1 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out with the spin refrigeration technique.
24. (once amended) The method of claim 1, wherein more than one assay is multiplexed and monitored by NMR spectroscopy and/or NMR imaging.
25. (once amended) The method of claim 1 wherein the assay is performed in a multiwell or multispot assay array.
26. (once amended) The method of claim 1 wherein step c) is performed by examining the assay reagent using both NMR spectroscopy to obtain more than one spectrum, and magnetic resonance imaging to obtain one or more discrete spectral location, and repeating the examination at least once so as to obtain quantitative information about kinetic or time-dependant alteration in chemistry, environment or structure of the assay reagent.
27. (once amended) The method of claim 1, wherein step c) is performed in an aerosol or flow-through device applied to aerosol droplets where the well, surface or container is used to contain the assay reagent.

28. (once amended) An *in vitro* assay kit for carrying out the assay method as defined in claim 1 which comprises: one or more assay reagents each containing at least one NMR active nucleus contained in a well or vial or other suitable container for carrying out the hyperpolarisation of step (b) of claim 1.
29. (once amended) The *in vitro* kit of claim 28 where the NMR analysis of step (c) is carried out in the same well, vial or container as the hyperpolarisation transfer is carried out.

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NMR SPECTROSCOPIC IN VITRO ASSAY USING HYPERPOLARIZATION

## TECHNICAL FIELD

5 The present invention relates to tailoring the shape of a magneto-resistive material, and more particularly to a design of the shape of the magneto-resistive material to obtain a new type of position sensitive sensor.

## BACKGROUND

10 The position of a moving object is often determined by means of the readout from a resistive sensor, usually of potentiometer type, which is mechanically connected to the object to be monitored.

15 In order to reduce the wear and thereby increase the reliability, it is desirable to eliminate the sliding friction encountered in the standard resistive sensors. Non-contact methods using e.g. inductively coupled coils is currently being introduced as replacement for the potentiometer sensors. However, these are more complex and therefore more expensive.

20 In recent years novel types of magneto-resistive materials with much higher sensitivity to moderate changes in magnetic fields have been found. These new materials showing giant magneto-resistance (GMR) or colossal magneto-resistance (CMR) make possible new types of position sensors.

25 In a document U.S. Patent No. 5,475,304 is disclosed a giant magneto-resistant sensor including at least one layered structure. The layered structure includes a ferromagnetic layer having a fixed magnetic state, a second, softer magnetic layer, and a metal layer interposed between and contacting these two layers. The sensor also includes one or more indexing magnets for inducing a domain wall, at a measured position, between regions of nonaligned magnetic fields in the softer magnetic layer. By  
30 measuring the resistance across the magneto-resistant sensor a displacement of one workpiece, carrying the sensor, will be measured relative to another workpiece carrying an inducing means.

nuclear polarisation of an assay reagent comprising at least one NMR active nucleus other than the noble gas. The hyperpolarisation of the assay reagent may also be achieved by using an artificially enriched hyperpolarised noble gas, preferably  $^3\text{He}$  or  $^{129}\text{Xe}$ .

5 Alternatively, hyperpolarisation may be imparted to atoms of significance in biological systems (e.g.  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{31}\text{P}$ ,  $^{29}\text{Si}$ ,  $^{19}\text{F}$  and  $^1\text{H}$  isotopes) by thermodynamic equilibration at very low temperature, suitably below 1K, preferably as close to 0 K as possible, and in the presence of a high magnetic field ("Brute force").

10 A further alternative is that hyperpolarisation may be imparted by dynamic nuclear polarisation (DNP). In the solid phase, the material is mixed with a paramagnetic species (DNP agent), for example a transition metal ion such as chromium (V) or manganese (II) and/or a free radical generator or other particles having associated free electrons. The method utilises a moderate or high magnetic field and very low temperature, e.g. by carrying out the conversion in  
15 liquid helium and a magnetic field of about 1 T or above.

A further technique for imparting hyperpolarisation is para hydrogen induced polarisation which involves cooling hydrogen to a low temperature, e.g. 20 K or less, to give para hydrogen enriched hydrogen. This enriched hydrogen is then used to hydrogenate an unsaturated  
20 target organic molecule (containing NMR active nuclei) imparting a non-thermodynamic spin configuration to the target molecule.

A yet further method covered by the present invention for preparing hyperpolarised materials is spin refrigeration. With this technique, the assay reagent is doped with or intimately  
25 mixed with a suitable paramagnetic material in crystal form (e.g. crystalline powder) with a symmetry axis of order three or more. One advantage with this technique is that there is no need for a uniform magnetic field since no resonant excitation field is applied. The sample is rotated to bring the electron paramagnetic resonance into contact with the nuclear spins, which are then cooled. The rotation is repeated until the nuclear spin polarisation is steady.

30 The present invention can give the same information about the target compared to any previously known NMR method, but with the advantage of increased sensitivity. A further



advantage of this invention is that assay reagent containing an NMR active nucleus may in many cases, provide the same information previously provided by corresponding  $^{14}\text{C}$ -labelled compounds, whilst being free from the problems associated with radioactive isotopes.

5 One further advantage according to the present invention is the increased signal-to-noise ratio. Another improvement with the present invention is that the time required to perform the assay is in general much shorter than the previously known methods. These improved parameters/results may be expressed as a "shortening effect", being the improvement of signal-to-noise ratio per unit time, and will be discussed further.

10 Yet another advantage compared e.g. with assays using fluorescent reagents is that there is no need to add an additional chemical component to the assay reagent to assist detection. There is always a disadvantage with techniques such as the fluorescent methods because the additional chemical component may influence the measurement.

15 The present invention provides an *in vitro* assay method which comprises:

- 20 a) using an assay reagent containing at least one NMR active nucleus to perform an assay, and  
b) hyperpolarising at least one NMR active nucleus of the assay reagent;

Wherein steps a) and b) are performed simultaneously or sequentially in either order, and

- c) analysing the assay reagent and/or the assay by NMR, and  
d) optionally using the NMR data obtained in step c) to generate further assay result(s).

25 As used herein, NMR active nuclei are those having non-zero nuclear spin and include  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{19}\text{F}$ ,  $^{29}\text{Si}$ ,  $^{31}\text{P}$  and/or deuterium. Of these,  $^{13}\text{C}$  and  $^{15}\text{N}$  are preferred and  $^{13}\text{C}$  is particularly preferred. Preferably the assay reagent for use in the assay according to this invention comprises an artificially-enriched abundance of an NMR active nucleus.

30 In a further preferred embodiment of the invention, the enriched compound comprises the artificially enriched NMR active nuclei, e.g.  $^{13}\text{C}$ , at one specific position. Alternatively, in another preferred embodiment the compound comprises enriched NMR active

nuclei in 1-10 defined positions. A further alternative embodiment of the present invention is to have the assay reagent uniformly labelled with artificially enriched NMR active nuclei.

An assay reagent is a substance or compound that takes part in an assay, by being introduced as an initial reagent or by being formed *in situ* and perhaps transiently during the assay, or by being formed as a product of the assay. An assay is a test performed partly or wholly *in vitro* in which a physical or chemical change involving a biological species is observed. This change may have occurred both *in vivo* and *in vitro*. A biological species is one which is present in living systems or which is introduced into and is reactive with such systems. Preferred assay methods covered by this invention are related to biological macromolecules such as proteins (e.g. enzymes, receptors, DNA, and RNA binding proteins, carrier proteins), oligonucleotides (e.g. DNA and RNA probes, DNA and RNA consensus sequences), macrocyclic molecules (e.g. cyclodextrin) carbohydrate macromolecules and lipids.

Many assays involve a reaction in which a chemical bond is broken. According to another embodiment of the present invention, the assay reagent is an organic compound comprising one or more NMR active nuclei wherein these nuclei are associated with a bond which is broken during the course of the assay.

According to another embodiment of the present invention, the assay reagent contains two or more different types of NMR active nuclei, e.g. both  $^{13}\text{C}$  and  $^{15}\text{N}$ . Each active nucleus produces a distinct NMR spectrum and when the assay method is performed its results in changes to the chemical and/or physical environment of the nucleus. The changes to the environment are mirrored by spectral changes, which can be monitored.

The degree of hyperpolarisation of the NMR active nucleus covered by this invention is in excess of 0.1 %, more preferably 1 % and even more preferably at least 10 % above the equilibrium population of the excited state.

Surprisingly, assay methods where even smaller enhancement is achieved may effectively be performed due to the shorter time needed for the total assay measurement. One

important aspect of the present invention is thus an assay wherein the time required to give a defined signal-to-noise is considerably shortened by the use of this hyperpolarisation technique compared to known assay techniques without hyperpolarisation. The shortening effect is expressed as the improvement of signal-to-noise ratio per unit time, dB  $\sqrt{\text{Hz}}$ . This effect is preferably a factor of 10 or more, more preferably a factor of 25 or more and even more preferably a factor of 50 or more. In some embodiments, this effect is particularly a factor of 200 or more or even a factor of 1000 or more.

The assay can be carried out with the NMR active nucleus in the assay reagent already hyperpolarised. Alternatively, the assay may be carried out and the NMR active nucleus subsequently hyperpolarised prior, or at the same time, as the assay/assay reagent is analysed by NMR spectroscopy. Whilst the first arrangement enables real time studies of the assay to be carried out, this is often not necessary and, in these circumstances, the second method is very useful. As hyperpolarisation of the NMR active nucleus will sometimes be carried out at a low temperature, e.g. 20 K or less, the assay can be started and then effectively frozen by lowering the temperature. The assay/assay reagent is then hyperpolarised and analysed by NMR spectroscopy. By carrying out this process a number of times, either on the same assay or on parallel assays, a series of "snap-shots" of how the assay is proceeding may be obtained.

When hyperpolarisation is effected by exchange in solution phase, the hyperpolarising agent can be introduced as one batch, continuously or intermittently. Some conditions would lead to rapid disappearance of the hyperpolarisation. However, continuous or intermittent hyperpolarisation will give adequate signal intensity. Repeating the hyperpolarisation – acquisition sequence will also enhance the signal to noise ratio.

Agents, such as organic solvents, may in some situations be added to the assay, and/or to the NMR active nucleus if this is to be hyperpolarised prior to the assay, in order to prolong the life time of the hyperpolarised NMR active nucleus in the assay reagent, without interfering with the assay reagent and/or assay method.

Assays can be carried out by quantifying the appearance, or the continued presence, or the disappearance of spectral patterns. For example, on binding or hybridisation of an assay

reagent the chemical shift of the signals derived from the NMR active nucleus in the assay reagent will change. The different relaxation times of the different NMR active nuclei need to be taken into account if the quantification measurement is to be accurate.

5 It will be apparent to those skilled in the art that some NMR active nuclei, also referred to herein as hyperpolarisable atoms, retain their hyperpolarisation for a longer period than others at a given set of physical parameters. Thus, the order in which steps (a) and (b) of the method are carried out may, to some extent, be determined by the choice of NMR active nucleus. Whilst there may be advantages in carrying out the hyperpolarisation of the assay reagent and then  
10 monitoring its NMR spectrum during the reaction, it is possible to "freeze" the reaction at any time. This may be achieved by reducing the temperature after the assay reagent has been added and then hyperpolarising the NMR active nucleus and comparing the spectra obtained with that of the assay reagent in a state where it has not undergone biological or chemical reaction(s).

15 As used herein, NMR active nuclei are those having non-zero nuclear spin and include  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{19}\text{F}$ ,  $^{29}\text{Si}$ ,  $^{31}\text{P}$  and deuterium. Of these,  $^{13}\text{C}$  and  $^{15}\text{N}$  are preferred and  $^{13}\text{C}$  is particularly preferred.  $^{13}\text{C}$  is present at a natural abundance (relative to  $^{12}\text{C}$ ) of about 1%. Just as the labelling of organic compounds with radioactive  $^{14}\text{C}$  is widely practised, so compounds, e.g. organic compounds can be labelled or enriched with  $^{13}\text{C}$ , either generally or at specific positions  
20 in the molecule. Preferably, the organic compounds for use in the assay according to this invention comprise an artificially-enriched abundance of  $^{13}\text{C}$ , either generally or at least in one specific position, at an abundance of at least 5%, suitably at least 10%, more suitably at least 50%, preferably at least 75%, more preferably at least 90% and ideally at approaching 100%.

25 The present invention also covers the use of compounds comprising an artificially-enriched abundance of  $^{15}\text{N}$  of at least 1%, suitably at least 5%, more suitably at least 10%, preferably at least 50% and more preferably at least 75% or more, and ideally at approaching 100%.

30 For assay reagents comprising  $^{29}\text{Si}$  the preferred level of artificially enriched abundance is at least 10% and more preferred at a level of 50% or more, even more preferably at least 75 % or more and ideally at approaching 100 %.

For achieving as long  $T_1$  as possible, the enriched compounds in some methods covered by the invention are preferably those in which the NMR active nucleus is surrounded by a double bond or one or more non-MR active nuclei such as O, S and/or C. In some cases, nearby  
5 protons to the NMR active nucleus may be substituted by deuterium.

In one embodiment of the invention, step c) is performed by examining the assay reagent using both NMR spectroscopy to obtain spectral data from one or more discrete physical locations and repeating the examination at least once so as to obtain quantitative information  
10 about kinetic or time-dependant alteration in chemistry, environment or structure of the assay reagent.

Assays envisaged according to this invention include for example, competition assays (e.g. receptor-ligand antagonism, enzyme-substrate inhibitors, protein-protein interaction  
15 inhibitors), binding assays (e.g. receptor-ligand agonism, enzyme-substrate reactions, protein-protein interactions), immunoassays (e.g. for specific analytes), hybridisation assays (e.g. nuclease assays, mutation analysis, mRNA and DNA detection), tests involving cells, organs and/or whole organisms. Thus, the invention covers binding studies performed on tissue sections, cultured cells, cellular metabolites, micro-organisms and macro-organisms. Preferred examples  
20 are discussed in the following paragraphs. Labelling with an NMR active nucleus where each molecule may be labelled at one or more chemical positions, will allow unique NMR assignments of e.g. starting material, intermediates and products of a biological reaction. Thus dual, triple etc labelling experiments can be carried out and 'stop-flow' measurements made with identical chemical species. For example, theoretically, all the six carbon atoms in glucose could be  
25 individually or collectively replaced by  $^{13}\text{C}$ , so that one to six of the carbon atoms are  $^{13}\text{C}$  which can be hyperpolarised. Each hyperpolarised  $^{13}\text{C}$  will give rise to a chemical shift, which will be specific to that individual carbon and different to other  $^{13}\text{C}$  positions in the molecule, i.e. C-1 will be different from C-2, etc.

A non-exclusive list of the types of molecules into which NMR active nuclei may be incorporated includes:

(i) Amino acids

Amino acids contain carbon, nitrogen and hydrogen and therefore any amino acid can be labelled at a single or multiple positions with one or more different NMR-active nuclei.

(ii) Lipophilic compounds

These would contain fatty acids, phospholipids, glycerol, cholesterol and its esters, sphingosine and its esters. These contain carbon, hydrogen and in some cases nitrogen and/or phosphorus and can be labelled at a single or multiple positions with one or more different NMR-active nuclei.

(iii) Vitamins

These include the water-soluble and fat-soluble categories of essential nutrients. These contain carbon, hydrogen and in some cases nitrogen and/or phosphorus and can be labelled at a single or multiple positions with one or more different NMR active nuclei.

(iv) Nucleic acids etc

DNA contains the bases adenine, cytosine, guanine and thymine and their nucleosides and nucleotides. RNA contains the bases adenine, guanine, cytosine and uracil and their nucleosides and nucleotides. These contain carbon, hydrogen, nitrogen and in some cases phosphorus and can be labelled at a single or multiple positions with one or more different NMR-active nuclei.

In one preferred embodiment of the invention the hyperpolarisation transfer is achieved by using a hyperpolarised noble gas, or a mixture of such gases, to effect nuclear polarisation of an assay reagent comprising at least one NMR active nucleus other than the noble gas.

5

When the hyperpolarisation of the assay reagent is achieved by an artificially enriched hyperpolarised noble gas, the hyperpolarised noble gas is preferably  $^3\text{He}$  or  $^{129}\text{Xe}$ . Such isotopically enriched gases are now commercially available at high isotope purity and can be polarised to a high degree of hyperpolarisation. The hyperpolarised gas may, if desired, be stored  
10 for extended periods of time in the polarised state, by keeping the gas at very low temperatures, especially in a frozen form.

A hyperpolarised noble gas may be used in step b) of the present invention to effect nuclear polarisation of an assay reagent comprising at least one NMR active nucleus other than  
15 the noble gas. The hyperpolarised gas may be in the gas phase, condensed or may alternatively be liquid e.g. by being dissolved or emulsified in a lipophilic solvent such as a lipid or a fluorocarbon solvent, or in a suspension or a solid e.g. by being adsorbed or frozen on to a solid surface. In some cases, liposomes or microbubbles may encapsulate the hyperpolarised noble gas.

20 The assay reagent may be solid, semi-solid or fluid. A hyperpolarised gas may be bubbled into a fluid assay system. Alternatively, a hyperpolarised gas solution may be mixed with a fluid assay. The hyperpolarised gas may be cooled and/or maintained in a magnetic field to preserve the hyperpolarisation. Similarly the resulting assay reagent comprising at least one polarised NMR active nucleus may preferably be cooled and/or maintained in a magnetic field in  
25 order to preserve the polarisation and/or facilitate polarisation transfer.

One advantage with hyperpolarisation transfer by  $^3\text{He}$  or  $^{129}\text{Xe}$  is that these gases are essentially chemically inert and will not adversely affect the assay reagent or the assay. In addition, as in gaseous form,  $^3\text{He}$  and/or  $^{129}\text{Xe}$  are easily separated from the assay medium,  
30 permitting facile repeat studies.

In one embodiment, a flow of hyperpolarised gas in the liquid state at elevated pressure and/or low temperature is passed through a column of the assay reagent. The gas may be pumped off and the process repeated until a suitable level of polarisation is achieved.

Alternatively, a hyperpolarised gas is frozen/crystallised on the solid/frozen surface of the solid assay reagent. This compound may preferably have been prepared with as large a surface area as possible, e.g. as a finely divided powder.

In some cases, it is desirable to remove part of or substantially the whole of the hyperpolarisable gas from the assay reagent/system as rapidly as possible. If desired, the gas may be reused which may be important due to the expense of isotopically-enriched noble gases. Many physical and chemical separation or extraction techniques known in the art may be employed to effect rapid and efficient separation of the hyperpolarised gas and the assay system.

In one embodiment of the invention when performed with the assay reagent in the solid phase, it is especially important that the content of  $^{131}\text{Xe}$  should be as low as possible. The preferred content of  $^{131}\text{Xe}$  is thus below 0.5 % of the total Xe content, and more preferably below 0.05 %.

In a further aspect, the present invention provides a method for optimising the polarisation enhancement factor when the assay reagent is hyperpolarised by a noble gas in solution. Thus the enhancement of the target nuclear spin can be optimised by slowing the dynamics of the molecules (atoms) in the solution. The dynamics can be slowed down, e.g. by increasing the viscosity of the solvent. The polarisation enhancement factor may also depend on the concentration of the noble gas in the solution and the enhancement factor may be optimised further by adjusting the pressure and temperature.

The relaxation mechanisms and also the relaxation of the target nucleus are partly functions of the viscosity of the solvent. For a specific system of interest we may choose the optimal viscosity of the medium that will lead to the maximal polarisation enhancement factor of the target nucleus. The viscosity is determined by choice of solvent and temperature. Preferably, the viscosity should be at least 1000 mPs, more preferably at least 10000 mPs and especially preferably at least 100000 mPs.



In one embodiment of the invention, when the polarisation transfer occurs in solution, the pressure of xenon is as high as possible, preferably higher than  $5 \times 10^5 \text{ N/m}^2$  (5 bar), more preferably higher than  $5 \times 10^6 \text{ N/m}^2$  (50 bar), even more preferably higher than  $1 \times 10^7 \text{ N/m}^2$  (100 bar) and particularly higher than  $2 \times 10^7 \text{ N/m}^2$  (200 bar). However, the pressure must never be so high so that the biological molecule will be totally or partly adversely effected.

It is preferred that the solvent comprises as few atoms which possess magnetic moment as possible and is as low magnetogyric ratio as possible. The transfer of polarisation in a highly viscous medium may be followed by solution spectroscopy under high-viscosity conditions (broad lines).

Alternatively, the viscosity may be lowered prior to spectroscopy, either by a change in temperature or by a change in the chemical composition of the solvent. If the high-viscosity medium is formed by a pH-sensitive gel-forming agent, then the viscosity might be lowered e.g. by a change in pH. Changes of temperature, ion-strength as well as the use of specific additives may also be considered.

In a further embodiment, the present invention provides a method wherein the hyperpolarisation transfer is effected by use of a very high field and with very low temperature (Brute force). The magnetic field strength used should be as high as possible, suitably higher than 1T, preferably higher than 5T, more preferably 15T or more and especially preferably 20T or more. The temperature should be very low e.g. 4.2K or less, preferably 1.5K or less, more preferably 1.0K or less, especially preferably 100 mK or less.

25

US 5479925 discloses a method for generating MR angiograms in which a contrast agent is passed through a small, high field polarising magnet *in vitro* in order to generate a high longitudinal magnetisation in the agent prior to its administration to the subject. However, there is no mention of the use of an enriched NMR active nucleus. When this Brute force method is used, and thermodynamic equilibrium is attained, all nuclei in the assay reagent will be highly polarised relative to room temperature and to normal magnetic fields used in MRI.

30

A major practical problem when using this technique is the time required for the thermal equilibrium to occur. However, the Brute force embodiment may be modified in order to solve this problem as described below.

5 It is possible to use a technique of low-field matching to increase the relaxation rate and the degree of polarisation of the nuclear spins in solids at low temperature. This has the additional advantage that equipment used in the Brute force polariser does not need to possess any radio frequency electronics.

10 A way of speeding up the polarisation of the NMR active nuclei, at least for  $^{13}\text{C}$  and  $^{15}\text{N}$  and at the same time obtaining a better polarisation is to use cross-polarisation from the quickly relaxing proton to the slowly relaxing carbon, a method routinely used in solid-state NMR spectroscopy. The situation may be further improved by utilising the procedure of spin locking under Hartman-Hahn conditions. However, radiofrequency electronics are required and  
15 furthermore the homogeneity of the magnetic field must be high enough to allow precise pulse angles. A simplified method to allow for thermal contact between the protons and the NMR active nucleus (e.g.  $^{13}\text{C}$  or  $^{15}\text{N}$ ) is to remove the assay from the magnet for a fraction of a second and repeat this procedure after the protons have repolarised, successively building up the polarisation until the spin-temperature of the two nuclei become the same.  
20

A further improvement of the Brute force embodiment of this invention is to optionally expose the assay system to a relaxation shortening effect in order to attain thermodynamic equilibrium at said low temperature. The relaxation shortening effect may be provided by exposure to field cycling to a field allowing cross polarisation, gradually increasing  
25 the magnetic field at such a rate that the increase in polarisation of the assay reagent is maximised. This effect may also be achieved by adding magnetic material to the assay reagent during the period when the assay reagent is exposed to low temperature.

In a further embodiment, the present invention provides a method for the polarisation  
30 transfer using the DNP method effected by a DNP agent, to effect nuclear polarisation of an assay reagent comprising at least one NMR active nucleus. In the solid phase, there are two aspects of DNP, namely "the solid effect" and the thermal mixing.

Most known paramagnetic compounds may be used as a "DNP agent" in this embodiment of the invention, e.g. transition metals such as chromium ions or organic free radicals such as nitroxide radicals and trityl radicals (WO 98/58272). Where the DNP agent is a paramagnetic free radical, the radical may be conveniently prepared *in situ* from a stable radical precursor by a radical-generating step shortly before the polarisation, or alternatively by the use of ionising radiation. Energy, normally in the form of microwave radiation, is provided in the process which will initially excite the paramagnetic species. Upon decay to the ground state, there is a transfer of polarisation to an NMR active nucleus of the target material. The method may be conveniently carried out by using a first magnet for providing the polarising magnetic field and a second magnet for providing the primary field for MR spectroscopy/imaging.

In some cases, the radical will be non-reusable and may conveniently be discarded after use. Many physical and chemical separation or extraction techniques are known in the art, which may be used if it is desirable to remove the DNP agent from the assay system in a rapid and/or efficient separation step. Magnetic properties may e.g. be used to achieve the separation. It is particularly preferred to use a heterogeneous system, e.g. a two-phase liquid, a solid in liquid suspension or a high surface area solid substrate within a liquid. For any heterogeneous system, separation may be achieved by e.g. filtration, decanting, chromatographic or centrifugal methods.

In a further embodiment, the present invention provides a method wherein the polarisation transfer is achieved by exposing the assay reagent to para hydrogen-enriched hydrogen gas in the presence of a suitable catalyst. The assay reagents suitable for use are prepared from precursors which are able to be hydrogenated and which will therefore typically possess one or more unsaturated bonds, e.g. double or triple carbon-carbon bonds.

Hydrogen molecules exist in two different forms, para hydrogen ( $p\text{-H}_2$ ) where the nuclear spins are anti parallel and out of phase (singlet state) and ortho hydrogen ( $o\text{-H}_2$ ) where the spins are parallel or anti parallel and in phase (triplet state). At room temperature, the two forms exist in equilibrium with a 1:3 ratio of para:ortho hydrogen. However, preparation of para hydrogen enriched hydrogen can be carried out at low temperature, 160K or less, in the presence of a catalyst. The para hydrogen formed may be stored for long periods, preferably at low

temperature, e.g. 18-20K. Alternatively it may be stored in pressurized gas form in containers which have an inner surface which is non-magnetic and non-paramagnetic.

When the p-H<sub>2</sub> molecule is transferred to the precursors of the assay reagent (by  
5 means of catalytic hydrogenation with e.g. (PPh<sub>3</sub>)<sub>3</sub>RhCl), the proton spins remain anti parallel  
and begin to relax to thermal equilibrium with the normal constant T<sub>1</sub> of the hydrogen in the  
assay molecule. However, during relaxation some of the polarisation may be transferred to  
neighbouring nuclei by pulse sequence (Progress in Nuclear Spectroscopy, 31, (1997), 293-315),  
low field cycling or other types of coupling. The presence of the NMR active nucleus as e.g. <sup>13</sup>C  
10 (and <sup>15</sup>N etc) with a suitable substitution pattern close to the relaxing hydrogen may lead to the  
polarisation being trapped in the slowly relaxing <sup>13</sup>C (or <sup>15</sup>N etc) resulting in a high enhancement  
factor.

A further hyperpolarisation transfer embodiment of this invention is the spin  
15 refrigeration method. This method covers spin polarisation of a solid assay by spin refrigeration  
polarisation. The assay is doped with or intimately mixed with a suitable paramagnetic material  
such as Ni<sup>2+</sup>, lanthanide and actinide ions in crystal form with a symmetry axis of order three or  
more. The instrumentation is simpler than that required for DNP with no need for a uniform  
magnetic field. The process is carried out by physically rotating the sample around an axis  
20 perpendicular to the direction of the magnetic field. The prerequisite for this to work is that the  
paramagnetic species has a highly anisotropic g-factor.

Hybridisation assays are very widely used for sequencing and for detection of point  
or deletion mutations in nucleic acids. When a conventionally labelled polynucleotide probe is  
25 hybridised with a polynucleotide target, analysis of the melting temperature or other property of  
the hybrid can give some limited information about the nucleotide sequence of the target.

The present invention can give the same information about the target compared to  
any previously known NMR methods available, but with the advantage of increased sensitivity. A  
30 polarised NMR active nucleus generates an NMR spectrum which is dependent on its  
environment, i.e. the atoms surrounding the NMR active nucleus, both intramolecular (atoms  
within the same molecules as the NMR active nucleus) and intermolecular (atoms in the other

molecules nearby the NMR active nucleus). The environment thus extends beyond the labelled molecule itself to other molecules in the immediate vicinity. Thus for example, a nucleotide labelled with polarised NMR active nucleus, e.g.  $^{13}\text{C}$  and/or  $^{15}\text{N}$ , when incorporated into a single stranded polynucleotide chain, can give information about two or more adjacent nucleotide  
5 residues in the chain. When that labelled polynucleotide probe is hybridised with a polynucleotide target, NMR spectroscopic analysis of the NMR  $^{13}\text{C}$  label can give information about the complementary nucleotide residue in the target.

In one embodiment of the present invention, comparative and/or parallel testing is  
10 performed to maximise the information available from the NMR measurements.

Biological macromolecules such as nucleosides or nucleotides or nucleotide analogues can readily be enriched with a NMR active nucleus, e.g.  $^{13}\text{C}$  and/or  $^{15}\text{N}$  at one or several specified points in the molecule. Polarisation of the NMR active nucleus, e.g.  $^{13}\text{C}$ ,  
15 preferably by contact with a hyperpolarised noble gas, may be effected either before, during or after incorporation of the monomer into a polynucleotide; and before, during or after hybridisation of that polynucleotide with a complementary strand.

Figure 1 demonstrates a hybridisation assay in which the use of an oligonucleotide or  
20 polynucleotide is used to detect the presence of single nucleotide polymorphisms (SNPs) in a gene, or fragment of a gene. An oligonucleotide or polynucleotide probe is prepared in which one or more of the atoms has been replaced by a hyperpolarisable isotope, e.g.  $^{13}\text{C}$ ,  $^{15}\text{N}$  or  $^1\text{H}$ . This probe is then hybridised to the gene or the gene fragment. The probe will be "targeted" to information-rich parts of the gene and may be selected so that the probe binds only to that part of  
25 the DNA containing a specific mutation, or, potentially, more than one mutation. If desired, a set of probes, each probe containing a hyperpolarisable isotope, can be added to a gene or gene fragment, each probe being targeted to a different part of the gene/gene fragment. As each probe will have a characteristic chemical shift by NMR spectroscopy, the spectrum of the mixture of the probes with the target can be taken and resolved to indicate which probes have bound and  
30 which have not.

The probe may be polarised before, during, or after hybridisation and a determination

carried out by NMR of whether a shift has occurred in the signal obtained from the hyperpolarised isotopic atom(s). If a shift has occurred, then the probe is (by inference) in a different chemical environment indicating hybridisation. Clearly information can be obtained from both positive and negative results, e.g. a probe could be constructed from the “natural” gene, a naturally occurring DNA sequence, and if results indicate that this has failed to bind, probes could be tested containing anticipated mutations. This technique facilitates itself to use of an array-type format in which a number of hyperpolarisable probes are used in the assay which each vary by one nucleotide. The identity of the SNP can be determined by the hybridisation pattern of the probes to the gene/gene fragment.

As mentioned earlier, many assays involve a reaction in which a chemical bond is broken. According to one embodiment of the present invention, the assay reagent is an organic compound comprising one or more NMR active nuclei associated with a bond which is broken during the course of the assay. In the case of a single NMR active nucleus, this is located preferably at the actual site of the breaking of the chemical bond such that the change in local environment of the active nucleus subsequent to the bond breaking will give rise to a significant change in the spectrum of the NMR active nucleus. The NMR spectra of two or more active nuclei will be different, depending on whether they are present within the same molecule or in different molecules. When two or more NMR active nuclei are in an appropriate proximity to one another they are said to be spin coupled. This gives rise to a distinct NMR spectrum which can be monitored. It is therefore possible to analyse by NMR spectroscopy the rate and extent of the bond breaking by the disruption of the spin coupling. In this and other assays, the assay reagent may be analysed repeatedly by NMR spectroscopy at known time intervals so as to generate information about a change over time of the assay reagent.

Figure 2 demonstrates a proteolysis assay. The starting substrate for the reaction contains two hyperpolarisable isotopes, in this case  $^{13}\text{C}$ , which are sufficiently close together, either by virtue of being reasonably adjacent in the chain of amino acids comprising the molecule, or by the 3-dimensional conformation of the molecule held in a “conformational lock”. In these situations, NMR spectra J coupling (scalar coupling) of the signal occurs and the NMR spectra of the molecule is recorded.

The molecule is then brought into contact with an enzyme capable of altering the chemical composition of the substrate. If cleavage occurs between the amino acids containing the hyperpolarisable isotopic atoms, then the J coupling and the chemical shift values change which will be observed by NMR spectroscopy and/or NMR imaging. Two new spectra will appear, one  
5 for each of the individual cleavage products. If there is no cleavage, the original spectrum remains.

A similar assay can be carried out where the starting substrate is a chain of nucleotides and the cleavage enzyme an endonuclease.

10 In another aspect of the invention, an assay reagent may be administered to a macro-organism, e.g. a human or animal, and NMR spectroscopic analysis performed of blood, excreta, e.g. urine, faeces or breath, or samples of the macro-organism.

15 In yet another aspect of the invention, an assay reagent may be used in binding studies on bacteria or other eukaryotic or prokaryotic micro-organisms or cultured cells.

Assays according to one embodiment of this invention may conveniently be carried out in multiwell plates. An assay reagent in each well may e.g. be hyperpolarised by contact with a  
20 hyperpolarised noble gas, prior to addition of other assay reagents. Alternatively, an assay reagent in bulk may be hyperpolarised with a hyperpolarised noble gas prior to being dispensed into individual wells of a multiwell plate. In many cases, assays can be performed in a homogenous mode, that is to say without the need for a separation step to remove one fraction of the labelled reagent.

25 In addition, in cases where the spectra of the  $^{13}\text{C}$  labelled assay components are distinct from one another, more than one assay may be performed and simultaneously monitored in a single well or spot of a multi-assay array. This would allow multiplexing of several related or unrelated assays in parallel within a single well or spot in a multi-assay array which is either  
30 ordered or random. In addition the technique may be applied to aerosol droplets where no well, container or surface is used to contain the assay and to analysis of samples in flow-through devices.

Figure 3 illustrates how the incorporation of a material (for example an amino acid) into a cell can be measured. The material incorporates a hyperpolarisable isotopic atom, in this case  $^{13}\text{C}$ . Its NMR spectrum in a hyperpolarised state in the media used for the experiment is recorded. If the material crosses the cell membrane then the environment in which the material finds itself will change and this will affect the NMR chemical shift of the material. The precise chemical shift will depend on the environment of the material within the cell, for example it may be possible to identify whether it has crossed into the cell nucleus. Alternatively, the material may be bound to the surface of the cell, again a different spectrum will result. In addition, metabolites that contain a hyperpolarisable isotopic atom may be detected either inside the cell or after they are excreted from this. The spectra obtainable on these metabolites can be used for their identification and/or to give information on their structure.

In one embodiment of the present invention, the assay is performed at a relatively cold temperature. However, in some situations the assay is carried out at room temperature.

It is important that the probes, vials, coils etc are coated or made of materials which do not induce loss of polarisation, such as para-magnetic nuclei. Preferred such materials are e.g. plastic, aluminium, Teflon and glass (with low iron content) materials. A further embodiment of the method according to the invention is thus the use of materials such as aluminium, plastic, glass and/or Teflon for the wells, vials, containers and any coils. A metal may also be used coated with a non-para magnetic oxide layers (e.g. Ti, Mg or Ag).

In one preferred embodiment of the invention, the assay is carried out in an NMR tube, with a gas-tight seal, permitting the addition (and/or removal) of a hyperpolarised gas to (and/or from) the assay reagent.

A variety of NMR spectroscopy and/or NMR imaging manipulation methods may be used, e.g. magic angle spinning and pulse sequence like WAHUHA or MLEV-8 to obtain high resolution spectrum when the assay reagent is a solid or semi-solid state.



A further embodiment of the present invention is an *in vitro* kit for carrying out the assay method as defined. The kit comprises a well, vial or any other suitable container comprising one or more assay reagents optionally together with additives wherein the hyperpolarisation transfer occurs. One embodiment of the invention concerns an *in vitro* kit where the NMR analysis of step ( c ) of claim 1 is carried out in the same well, vial or container as the polarisation transfer is carried out.

The invention is illustrated with reference to the following non-limiting example. Modifications of the method according to this example include the addition of the noble gas directly into the spectrometer and the use of different pulse techniques.

#### Example 1.

Polarisation transfer from hyperpolarised  $^{129}\text{Xe}$  to the singly labelled peptide AcYRARV(F,  $^{13}\text{C}$ -amide)FVRAAK-NH<sub>2</sub>

Hyperpolarized  $^{129}\text{Xe}$  was generated by optical pumping as described by B.Driehuys et al., Appl.Phys.Lett. 69 (12), 1996. The isotopic composition of the gas was 80%  $^{129}\text{Xe}$  and 0.25%  $^{131}\text{Xe}$  (the rest non-magnetic isotopes of Xe). The degree of polarization was estimated to be  $10\% \pm 3$ .

The freeze-dried peptide (3.4 mg) was placed in an ordinary 5 mm thin-walled NMR-tube. The glass tube was connected to the outlet of the polarizer by means of 60 cm of plastic tubing. The tube was evacuated and then filled with nitrogen four times.

The hyperpolarized gas was generated and collected on a cold finger at liquid nitrogen temperature in a holding field of 200 mT over a period of 15 minutes which is estimated to give a volume of 50 ml of Xenon at NTP. A narrow Dewar vessel with liquid nitrogen was placed in a magnet with a field strength of 0.3 T. The collected xenon was thawed and gradually refrozen on the peptide from the bottom and up by gradually lowering the tube into the liquid nitrogen bath. The system was then filled with helium to one atmosphere. The sample, with the plastic tubing still connected but open to the surroundings, in the Dewar in the 0.3 T magnet with the poles in horizontal configuration was then moved into the stray-field of the 7 T magnet (vertical polarity)

of an NMR-spectrometer. The sample was then rapidly transferred to the spectrometer and was in the process subjected to a minimum magnetic field of 0.3 mT.

A  $^{13}\text{C}$  spectrum was recorded with a spectral window of 100 kHz and a broad  $^{13}\text{C}$  signal  
5 was obtained. The sample was then left to polarize in the magnet and a background signal was recorded overnight, and care was taken to allow for full relaxation between the pulses.

The enhancement was measured to  $6\pm 1$  times the thermodynamic equilibrium at 7 T and 291 K.

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The time from the beginning of freezing the xenon in the NMR tube to the acquisition of the spectrum was 5 minutes.

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## C L A I M S

1. An *in vitro* assay method which comprises:
  - a) using an assay reagent containing at least one NMR active
  - 5 nucleus to perform an assay, and
  - b) hyperpolarising at least one NMR active nucleus of the assay reagent;  
wherein steps (a) and (b) are performed simultaneously or sequentially in either order, and
  - c) analysing the assay reagent and/or the assay by NMR and
  - 10 d) optionally using the NMR data obtained in step c) to generate further assay result(s).
2. The method of claim 1 wherein the NMR active nucleus is  $^{15}\text{N}$ ,  $^{19}\text{F}$ ,  $^{31}\text{P}$ ,  $^1\text{H}$ ,  $^{29}\text{Si}$  and/or  $^{13}\text{C}$ .
3. The method of claim 2, wherein the NMR active nucleus is  $^{15}\text{N}$  or  $^{13}\text{C}$ .
4. The method of any of claims 1 to 3, wherein the assay reagent is a compound which contains an artificially high concentration of an NMR active nucleus.
5. The method of claim 4, wherein the assay reagent contains an artificially high concentration in 1-10 defined positions.
6. The method of any of claims 1 to 5, wherein the assay reagent is an organic compound comprising one or more NMR active nuclei associated with a bond which is broken during the course of the assay.
7. The method of claim 6, wherein the assay reagent contains two or more NMR active nuclei and each NMR active nucleus produces a distinct NMR spectrum and when the

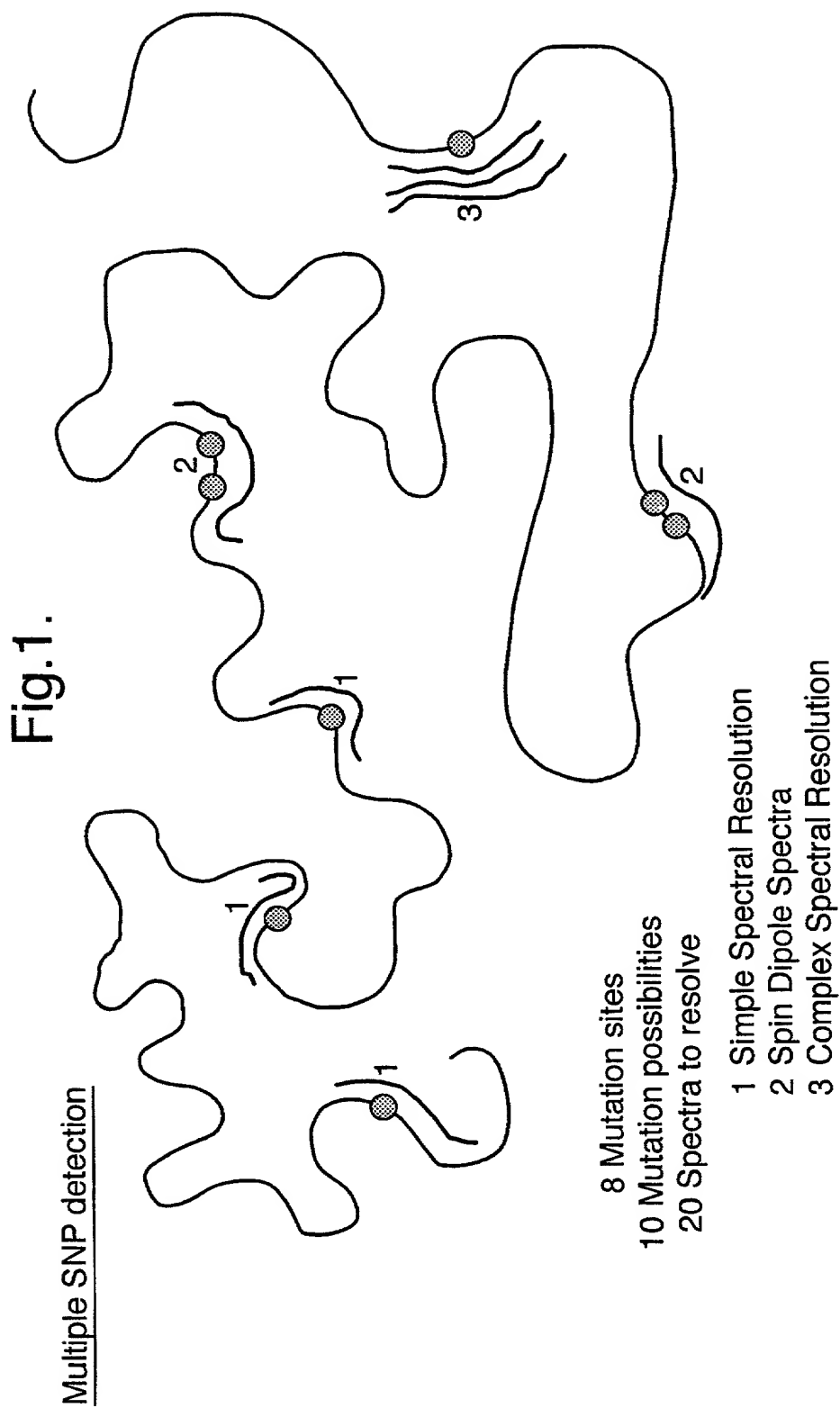
assay method is performed, it results in changes to the chemical and/or physical environment of the nucleus and this is mirrored by spectral changes which can be monitored.

- 5 8. The method of any claim 1-7, wherein the assay reagent is analysed repeatedly in step c) at known time intervals so as to generate information about a change with time of the assay reagent.
9. The method of any one claim 1 to 8, wherein the assay reagent is a Nucleotide, or  
10 nucleotide analogue, polynucleotide, amino acid analogue, polypeptide or protein.
10. The method of any one of claims 1 to 9, wherein the assay is a nucleic acid hybridisation assay.
- 15 11. The method of any one of claims 1 to 10, wherein the assay is a binding assay.
12. The method of claims 1 to 11, wherein the assay reagent is a compound specifically labelled with at least one NMR active nucleus and the assay reagent is administered to a micro-organism, macro-organism or cultured cells, cellular metabolites or an excretion  
20 product of the assay reagent are hyperpolarised and analysed by nuclear magnetic resonance spectroscopy, nuclear magnetic resonance imaging or both.
13. The method of claims 1 to 12, wherein the assay is a binding study performed using micro-organisms or cultured cells.
- 25 14. The method of claims 1 to 13 wherein the hyperpolarisation transfer is repeated to enhance the signal-to-noise ratio.
- 30 15. The method of claim 1 to 14 wherein the shortening effect as expressed by the improvement of signal-to-noise per unit time is a factor of 10 or more compared to known assay techniques without hyperpolarisation.

16. The method of claims 1 to 15 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer from a hyperpolarised noble gas, or a mixture of hyperpolarised noble gases.
- 5 17. The method of claim 16 wherein the noble gas is  $^{129}\text{Xe}$ .
18. The method of claim 16 wherein the noble gas is  $^3\text{He}$ .
19. The method of claims 16 to 18 wherein the hyperpolarisation is transferred by a  
10 hyperpolarised noble gas in solution and wherein the viscosity of the solution is at least 1000 mPs.
20. The method of claims 1 to 15 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer at a temperature of 4.2 K or less in  
15 the presence of a magnetic field of at least 1 T.
21. The method of claims 1 to 15 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by polarisation transfer using dynamic nuclear  
20 polarisation.
22. The method of claims 1 to 15 where the hyperpolarisation of the NMR active nucleus of the assay reagent is carried out by para hydrogen induced polarisation.
23. The method of claims 1 to 15 where the hyperpolarisation of the NMR active nucleus of  
25 the assay reagent is carried out with the spin refrigeration technique.
24. The method of claims 1 to 23, wherein more than one assay is multiplexed and monitored by NMR spectroscopy and/or NMR imaging.
- 30 25. The method of claims 1 to 24 wherein the assay is performed in a multiwell or multispot assay array.

26. The method of claims 1 to 25 wherein step c) is performed by examining the assay reagent using both NMR spectroscopy to obtain more than one spectrum, and magnetic resonance imaging to obtain one or more discrete spectral location, and repeating the examination at least once so as to obtain quantitative information about kinetic or time-dependant alteration in chemistry, environment or structure of the assay reagent.
27. The method of claim 1 to 26, wherein step c) is performed in an aerosol or flow-through device applied to aerosol droplets where the well, surface or container is used to contain the assay reagent.
28. An *in vitro* assay kit for carrying out the assay method as defined in claim 1 to 27 which comprises: one or more assay reagents each containing at least one NMR active nucleus contained in a well or vial or other suitable container for carrying out the hyperpolarisation of step (b) of claim 1.
29. The *in vitro* kit of claim 28 where the NMR analysis of step (c) of claim 1 is carried out in the same well, vial or container as the hyperpolarisation transfer is carried out.

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Fig.2.

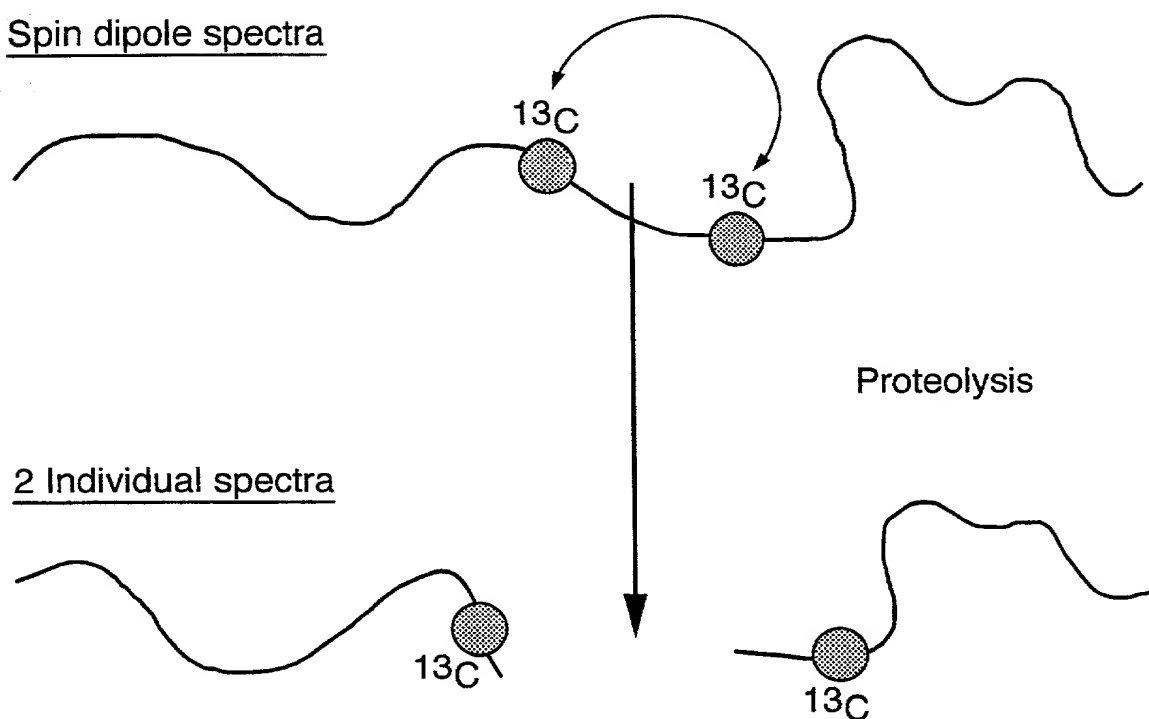
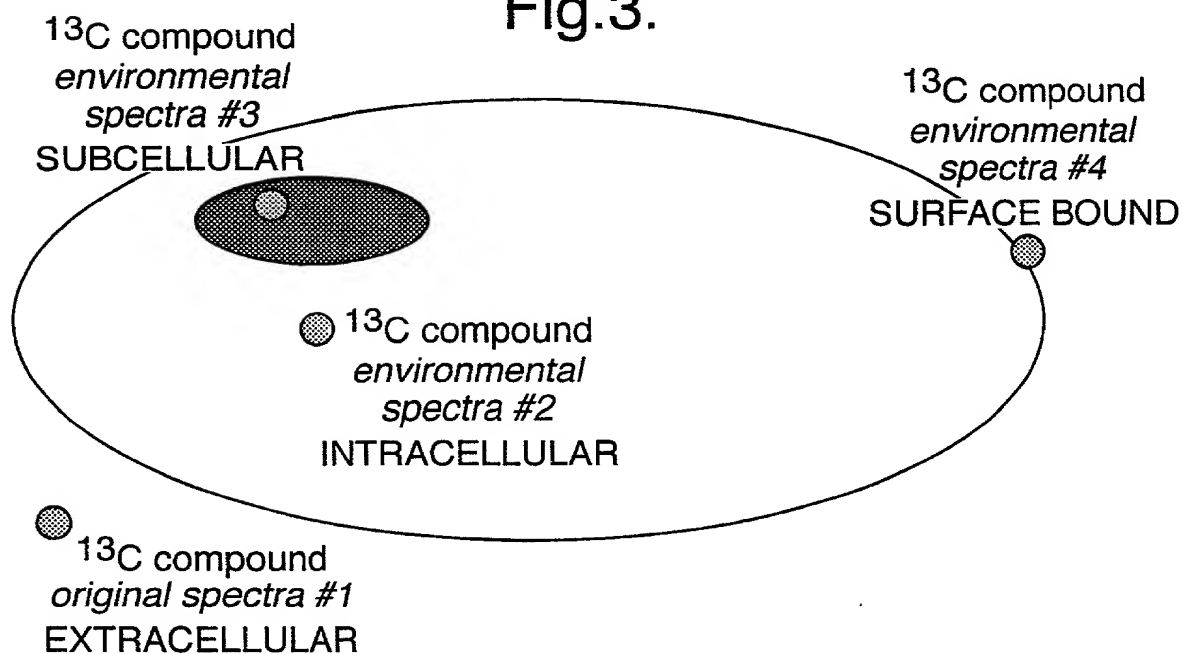
Spin dipole spectra2 Individual spectra

Fig.3.





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**DECLARATION FOR UTILITY OR  
DESIGN  
PATENT APPLICATION  
(37 CFR 1.63)**

☐ Declaration  
Submitted with Initial  
Filing  
OR  
☒ Declaration  
Submitted after Initial  
Filing (surcharge  
(37 CFR 1.16 (e))  
required)

Attorney Docket Number PA-9848

First Named Inventor Knox

**COMPLETE IF KNOWN**

Application Number 09 / 869,629

Filing Date 28-Jun-2001

Group Art Unit To be assigned

Examiner Name To be assigned

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**NMR Spectroscopic In Vitro Assay Using Hyperpolarization**

the specification of which (Title of the Invention)

☐ is attached hereto  
OR

☒ was filed on (MM/DD/YYYY) 06/28/2001 as United States Application Number or PCT International

Application Number 09/869,629 and was amended on (MM/DD/YYYY) (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
9828852.5	Great Britain	12/30/1998	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
9918096.0	Great Britain	08/02/1999	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
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☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below

Application Number(s)	Filing Date (MM/DD/YYYY)	<input type="checkbox"/> Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto

[Page 1 of 2]

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## DECLARATION

ADDITIONAL INVENTOR(S)  
Supplemental Sheet  
Page 1 of 2

Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any])		Family Name or Surname	
2-0 Neil		Cook	
Inventor's Signature	Date		
Residence: City	State	Country	GB
Post Office Address Tutshill Lodge, Beachley Road, Tutshill			
Post Office Address Chepstow, Great Britain NP6 7EG GBN			
City	State	ZIP	Country
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any])		Family Name or Surname	
3-0 Klaes		Golman	
Inventor's Signature	Date		
Residence: City	State	Country	SE
Post Office Address Nycomed Innovation AB, Ideon Malmo			
Post Office Address Per Albin Hanssons vag 41, S-205 12 Malmo Sweden SE			
City	State	ZIP	Country
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any])		Family Name or Surname	
4-0 Haukur		Johannesson	
Inventor's Signature	Date		August 27, 2001
Residence: City	State	Country	SE
Post Office Address Nycomed Innovation AB, Ideon Malmo			
Post Office Address Per Albin Hanssons vag 41, S-205 12 Malmo Sweden SE			
City	State	ZIP	Country

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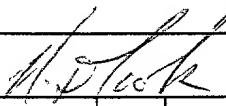
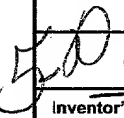
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## DECLARATION

ADDITIONAL INVENTOR(S)  
Supplemental Sheet  
Page 1 of 2

<b>Name of Additional Joint Inventor, if any:</b>				<input type="checkbox"/> A petition has been filed for this unsigned inventor			
Given Name (first and middle [if any])				Family Name or Surname			
Neil				Cook			
Inventor's Signature				20/8/01 Date			
Residence: City		Princeton		State		NJ	
				Country		USA	
Citizenship						GB	
Post Office Address		Amersham Pharmacia Biotech Inc, 800 Centennial Avenue					
Post Office Address		Piscataway, NJ 08855-1327, USA					
City				State			
				ZIP			
Country							
<b>Name of Additional Joint Inventor, if any:</b>				<input type="checkbox"/> A petition has been filed for this unsigned inventor			
Given Name (first and middle [if any])				Family Name or Surname			
Klaes				Golman			
Inventor's Signature				Date			
Residence: City				State			
				Country		SE	
Citizenship						DK	
Post Office Address		Nycomed Innovation AB, Ideon Malmo					
Post Office Address		Per Albin Hanssons vag 41, S-205 12 Malmo Sweden					
City				State			
				ZIP			
Country							
<b>Name of Additional Joint Inventor, if any:</b>				<input type="checkbox"/> A petition has been filed for this unsigned inventor			
Given Name (first and middle [if any])				Family Name or Surname			
Oksar				Axelsson			
Inventor's Signature				Date			
Residence: City				State			
				Country		SE	
Citizenship						SE	
Post Office Address		Nycomed Innovation AB, Ideon Malmo					
Post Office Address		Per Albin Hanssons vag 41, S-205 12 Malmo Sweden					
City				State			
				ZIP			
Country							

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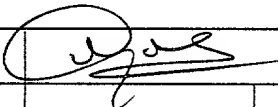
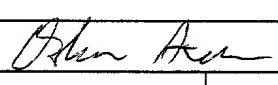
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## DECLARATION

ADDITIONAL INVENTOR(S)  
Supplemental Sheet  
Page 1 of 2

Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor			
Given Name (first and middle [if any])		Family Name or Surname			
Neil		Cook			
Inventor's Signature				Date	
Residence: City	State	Country	GB	Citizenship	GB
Post Office Address	Tutshill Lodge, Beachley Road, Tutshill				
Post Office Address	Chepstow, Great Britain NP6 7EG				
City	State	ZIP	Country		
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor			
Given Name (first and middle [if any])		Family Name or Surname			
Klaes		Golman			
Inventor's Signature				Date	8-8-2001
Residence: City	State	Country	SE	Citizenship	DK
Post Office Address	Nycomed Innovation AB, Ideon Malmo				
Post Office Address	Per Albin Hanssons vag 41, S-205 12 Malmo Sweden				
City	State	ZIP	Country		
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor			
Given Name (first and middle [if any])		Family Name or Surname			
Oksar		Axelsson			
Inventor's Signature				Date	3/8 2001
Residence: City	State	Country	SE	Citizenship	SE
Post Office Address	Nycomed Innovation AB, Ideon Malmo				
Post Office Address	Per Albin Hanssons vag 41, S-205 12 Malmo Sweden				
City	State	ZIP	Country		

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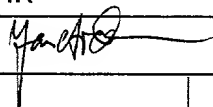
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## DECLARATION

ADDITIONAL INVENTOR(S)  
Supplemental Sheet  
Page 2 of 2

Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor			
Given Name (first and middle [if any])		Family Name or Surname			
Jan Henrik		Ardenkjaer-Larsen			
Inventor's Signature				Date	27-7-2001
Residence: City	State	Country	SE	Citizenship	DK
Post Office Address	Nycomed Innovation AB, Ideon Malmo				
Post Office Address	Per Albin Hanssons vag 41, S-205 12 Malmo Sweden				
City	State	ZIP	Country		
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor			
Given Name (first and middle [if any])		Family Name or Surname			
Inventor's Signature				Date	
Residence: City	State	Country		Citizenship	
Post Office Address					
Post Office Address					
City	State	ZIP	Country		
Name of Additional Joint Inventor, if any:		<input type="checkbox"/> A petition has been filed for this unsigned inventor			
Given Name (first and middle [if any])		Family Name or Surname			
Inventor's Signature				Date	
Residence: City	State	Country		Citizenship	
Post Office Address					
Post Office Address					
City	State	ZIP	Country		

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## DECLARATION — Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
PCT/GB99/04410	12/23/1999	

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02A attached hereto

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact with the Patent and Trademark Office connected therewith: ☒ Customer Number 22840 OR ☐ Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto

Direct all correspondence to: ☒ Customer Number 22840 OR ☐ Correspondence address below

Name					
Address					
Address					
City		State		ZIP	
Country		Telephone		Fax	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle if any)	Family Name or Surname
Peter	Knox

Inventor's Signature	<u>Peter Knox</u>	Date	<u>30.7.01</u>		
Residence: City	State	Country	GB	Citizenship	GB
Post Office Address	"Choppings", 34 Kings Road, Buckinghamshire				
Post Office Address	Chalfont St. Giles, Great Britain HP8 4HS				
City	State	ZIP		Country	

☒ Additional inventors are being named on the 2 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto